Reference: *Biol. Bull.* **195:** 308–318. (December, 1998)

Reproduction-Associated Immunoreactive Peptides in the Nervous Systems of Prosobranch Gastropods

JEFFREY L. RAM[1], CARLOS S. GALLARDO[2], MICHAL L. RAM[1], AND ROGER P. CROLL[3]

[1]*Department of Physiology, Wayne State University, Detroit, Michigan 48201;* [2]*Department of Zoology, Universidad Austral de Chile, Valdivia, Chile; and* [3]*Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada*

Abstract. Antibodies against reproductive peptides of *Aplysia* and *Lymnaea* were used to localize homologous immunoreactive peptides in the nervous systems of three prosobranch species: *Busycon canaliculatum, Concholepas concholepas,* and *Tegula atra.* Positive control experiments in *L. stagnalis* demonstrated the broad species range of the anti-egg-laying hormone (anti-ELH) antibody used in this study, and showed binding of anti-α-caudodorsal-cell peptide (anti-α-CDCP) to the same cells in cerebral and buccal ganglia. Dot immunoassays with synthetic ELH confirmed the reactivity and sensitivity (<0.1 μg) of the anti-ELH antibody. Experiments with preadsorbed antibody or no primary antibody confirmed its specificity.

In *B. canaliculatum,* clusters of more than 300 neuronal cell bodies immunoreactive to both anti-ELH and anti-α-CDCP were observed along the medial margins of left and right cerebral ganglia. Anti-α-CDCP reacted with additional small populations of cerebral ganglion neurons not stained by anti-ELH. Anti-ELH and anti-α-CDCP also reacted with overlapping but different small populations of neurons in buccal ganglia. In *C. concholepas* and *T. atra,* ELH-like immunoreactivity was found in cerebral ganglia, and in *T. atra* in fibers in the cerebral ganglia and cerebral-pedal connectives. Thus, cerebral ganglia are the major locus of the ELH-like immunoreactivity in prosobranchs.

Introduction

Prosobranch gastropods are the most ancient and numerous subclass of gastropod molluscs (Solem, 1991). Of the three extant subclasses, Prosobranchia evolved first—more than 400 million years ago—and radiated strongly; so the species in this subclass now occupy diverse habitats and thus exhibit an extraordinary variety of adaptations, including reproductive mechanisms. The two other gastropod subclasses, Opisthobranchia and Pulmonata, are derived from a common ancestor that diverged—first from the Prosobranchia, and from each other shortly thereafter—about 350 million years ago. Indeed, some classifications join the opisthobranchs and pulmonates under a single taxon known as Euthyneura (Haszprunar, 1988). The euthyneuran species are all hermaphrodites, in striking contrast to the diverse prosobranchs.

Prosobranchs generally have separate sexes, male and female, but sex reversal is known to occur in several species (*e.g., Crepidula fornicata,* Collin, 1995; *Busycon carica,* Castagna and Kraeuter, 1994). Fertilization is usually internal, and the females lay their fertilized eggs in protective egg capsules, but some species discharge gametes into the water column, where external fertilization takes place.

The great abundance, long evolutionary history, and diversity of the prosobranchs should make this taxon ideal for studies of the evolution and mechanisms of reproductive adaptations, from the molecular level to behavior. Unfortunately, relatively little is known about molecular mechanisms in prosobranchs, particularly as applied to the humoral regulation of reproductive behavior. The objective of the present study, therefore, was to investigate

Received 10 December 1997; accepted 29 September 1998.
E-mail: jeffram@med.wayne.edu

Abbreviations: CDC, caudodorsal cells; CDCH, CDC hormone; α-CDCP, alpha-CDC peptide; ELH, egg-laying hormone; PB, phosphate buffer; PFAPB, 4% paraformaldehyde in PB; TTBS, nonspecific blocker solution for dot immunoassay.

the localization of putative reproductive peptides in the nervous systems of a set of diverse prosobranchs, with the long-term goals of investigating roles of these peptides in the reproductive strategies of prosobranchs and, possibly, exploiting their function in mariculture.

A reproductive role for neuropeptides in prosobranch egg laying is supported by experiments in which nervous system extracts have been injected into mature females. Nervous system extracts elicit laying of eggs or egg capsules in the whelks *Busycon canaliculatum* and *Busycon carica* (Ram, 1977) and in the top snails *Gibbula cineraria, G. umbilicalis,* and *Monodonta lineata* (Clare, 1986, 1987). The active factors are protease sensitive (*B. canaliculatum,* Ram, 1977; *G. umbilicalis,* Clare, 1986), and in *B. canaliculatum* its molecular mass is about 5 kDa, as estimated by gel filtration (Ram *et al.,* 1982). These data are consistent with a molecule similar to a pair of homologous neuropeptides that cause egg laying in two euthyneuran species. These neuropeptides are egg-laying hormone (ELH) from the opisthobranch *Aplysia californica* (Kupfermann, 1967; Toevs and Brackenbury, 1969; Chiu *et al.,* 1979) and caudodorsal cell hormone (CDCH) from the pulmonate *Lymnaea stagnalis* (De Vlieger *et al.,* 1980; Ebberink *et al.,* 1985; Geraerts *et al.,* 1988). Some of us have speculated that the substances that activate egg laying in prosobranchs are also homologous with ELH and CDCH (Ram and Ram, 1990). To determine whether prosobranch nervous systems contain peptides homologous to the ELH/CDCH peptide family and to localize them if they are present, we have investigated the immunoreactivity of nervous tissues from several prosobranch species to antibodies to ELH and to alpha-CDCP (the latter is a small peptide derived from the CDCH precursor; Gerearts *et al.,* 1988).

The prosobranch species investigated here were *Busycon canaliculatum* (Neogastropoda, Melongenidae; whelk), *Concholepas concholepas* (Neogastropoda, Muricidae; loco), and *Tegula atra* (Archaeogastropoda, Trochidae; top snail). *B. canaliculatum* has been the subject of several previous studies of humoral control of egg laying in a prosobranch (Ram, 1977; Ram *et al.,* 1982); it is also the basis of a small, but important, fishery in Atlantic waters off the northeastern coast of the United States (Kaplan and Boyer, 1992). *C. concholepas* and *T. atra* are commercially important Chilean prosobranch species (Coloma, 1974; Osorio, 1979). *C. concholepas* is the most intensively studied Chilean prosobranch (for review, see Castilla, 1988), for which there is great concern about over-exploitation (Gallardo and Carrasco, 1996) and interest in its reproduction (Castilla and Cancino, 1976), development (DiSalvo and Carriker, 1994; Rodriguez *et al.,* 1995), and behavior (Serra *et al.,* 1997). As an archaeogastropod, *T. atra* adds phylogenetic diversity to the study; in contrast with the other two species, it exhibits external fertilization and a more primitive neuroanatomy.

We have determined that all three prosobranch species have anti-ELH immunoreactivity in clusters of cell bodies in the cerebral ganglia. In the cerebral ganglia of *B. canaliculatum,* immunoreactivity to anti-alpha-CDCP occurred in the same cells as immunoreactivity to anti-ELH, and it also appeared in several additional cells. Also in this species, anti-ELH and anti-alpha-CDCP immunoreactivity appeared in small overlapping, but distinct, populations of cell bodies in the buccal ganglia. The findings reported here provide a molecular and anatomical basis for investigations into the roles of molluscan reproductive peptides in prosobranch reproductive adaptations.

Materials and Methods

Animals

Experiments on *B. canaliculatum* were performed at Dalhousie University, Halifax, Nova Scotia, Canada. For Chilean species, two of the authors (JLR and MLR) traveled to Chile to collect specimens, and to dissect and fix nervous system preparations (as described below). Fixed material was brought back to Halifax for further processing and analysis. Positive controls on *L. stagnalis* utilized animals that were cultured at Dalhousie University in freshwater aquaria with continuously recirculated, filtered tap water, as previously described (Croll and Chiasson, 1989).

B. canaliculatum was obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, USA. Specimens were large (>12 cm, length from apex to siphon), and both males and females were used. Animals were maintained for up to 3 months in sea tables at approximately 10°C, and marine mussels were provided for food. *T. atra* was collected from tide pools near the field station of the Universidad Austral de Chile (Valdivia) located at Mehuin Bay, Chile (39°25′ S; 73°13′ W). *C. concholepas* was collected from tide pools at Playa Rosada, Chile (39°43′ S; 73°23′ W); other specimens of *C. concholepas* were obtained from A. Pinto of the Instituto de Fomento Pesquero, Castro, Chile (42°29′ S; 73°46′ W), where they were being grown by aquaculture. Both *C. concholepas* and *T. atra* were dissected shortly after collection.

Dissection, fixation, sectioning, and staining

Nervous systems of each of the above species were removed and further dissected, as needed, in either artificial saline (for *L. stagnalis;* Jansen and ter Maat, 1985) or filtered seawater (all other species). Ganglia were pinned out on Sylgard-coated dishes and cleaned of overlying connective tissue. The relative positions of ganglia were carefully noted so they could be identified accurately in sectioned material.

For *L. stagnalis*, the dissected preparations usually included cerebral, pedal, parietal, and buccal ganglia; cuts were made in the pedal commissure to produce an opened, flat ring of ganglia prior to fixation. For *B. canaliculatum* (Pierce, 1950; Bullock, 1965), cuts were made either between left pleural and left parietal (subesophageal) ganglia, or between cerebral ganglia prior to pinning out for dissection. The latter orientation is shown schematically in the Results to indicate the location of immunoreactive neurons. The nervous system of *C. concholepas*, like that of *B. canaliculatum*, consists of several rings of ganglia closely surrounding the esophagus (Fig. 1); thus, the dissection of the nervous system of *C. concholepas* was similar to that of *B. canaliculatum*. As a more primitive prosobranch, *T. atra* has a less compact set of ganglia than the other species. The nervous system of *T. atra* is almost identical to that of *Trochus* illustrated in Hyman (1967), which was used as a dissection guide. The anteriorly located pair of cerebral ganglia are connected by cerebral-pedal connectives to the pedal ganglia, which were easily identified by their location, shape, and the presence of statocysts. Most preparations included cerebral and pedal ganglia, the cerebral commissure, and the cerebral-pedal connectives, but in some preparations, other ganglia attached to these were also retained.

After dissecting and pinning out preparations in saline solution in the desired orientation, the solution was replaced with 4% paraformaldehyde in phosphate buffer (0.1 *M*, pH 7.4) (PFAPB) at 4°C. For experiments with *L. stagnalis* and *B. canaliculatum*, PFAPB was made fresh weekly and stored at 4°C. For the Chilean species, PFAPB was several weeks older and was maintained at room temperature for significant amounts of time during transport to and within Chile. Ganglia were fixed in PFAPB at 4°C for 24 h, then unpinned from the Sylgard and transferred to 70% ethanol for short- or long-term storage. Ganglia retained their shape and relative orientation after fixation. All preparations of *L. stagnalis* and *B. canaliculatum* were refrigerated while in 70% ethanol, and storage time ranged from 24 h to several days before further processing. Chilean specimens were maintained in 70% ethanol and refrigerated, when possible, for 3 to 4 weeks before further processing.

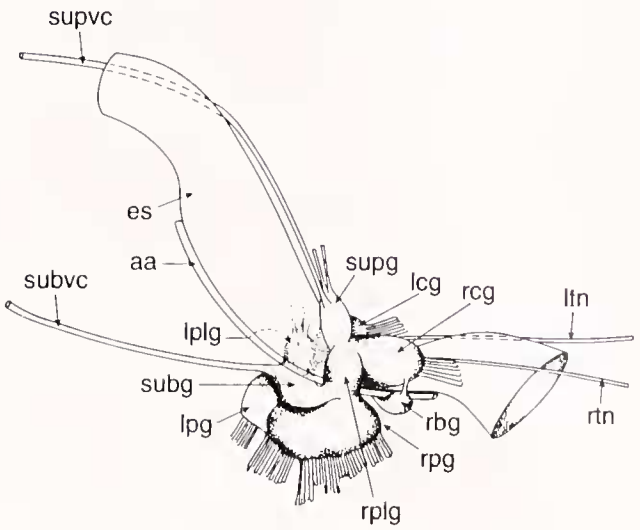

Figure 1. Diagram of the central nervous system of *Concholepas concholepas*, redrawn from original artwork by Huaquin (1966). Abbreviations: aa, anterior aorta; es, esophagus; lcg, left cerebral ganglion; lpg, left pedal ganglion; lplg, left pleural ganglion; ltn, left tentacle nerve; rbg, right buccal ganglion; rcg, right cerebral ganglion; rpg, right pedal ganglion; rplg, right pleural ganglion; rtn, right tentacle nerve; subg, subesophageal ganglion; subvc, subesophageal visceral connective; supg, supraesophageal ganglion; and supvc, supraesophageal visceral connective. Lplg and several nerves that are behind the esophagus are shaded as if seen through it. Subvc and supvc connect to the visceral ganglion (not shown).

Prior to cryosectioning, preparations were transferred to 30% sucrose in phosphate buffer (PB, 0.1 *M*, pH 7.4) and stored overnight at 4°C. Preparations were frozen in TissueTek in the cryostat at −15°C and sectioned at 30 μm. In some experiments, alternate sections were put on separate slides so that different antibodies or control procedures could be compared. Sections were dried, rinsed three times in PB, and incubated overnight at room temperature in primary antibodies (rabbit anti-ELH serum, 1:500 in 2% normal goat serum and 0.2% Triton X-100 in PB; or rabbit anti-α-CDCP, 1:500 in the same medium). They were then rinsed three times in PB, incubated in fluoroscein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (1:20, Bio/Can Scientific, Mississauga, Ont.) in the dark at 4°C overnight, rinsed three more times in PB, and then mounted in 75% glycerol in PB. Sections were viewed and photographed through a Leitz Aristoplan microscope for epifluorescence with I3 or L3 filter block.

The primary anti-ELH is an affinity-purified antibody made in rabbits by J. E. Blankenship and G. T. Nagle (Marine Biomedical Institute, University of Texas Medical Branch, Galveston, TX), who used as antigen a synthetic N-terminal fragment of *Aplysia* ELH (ISINQDLKAITDMLC; the C-terminal Cys was for coupling purposes and is not part of the N-terminal ELH sequence). The antibody was characterized as specifically staining *Aplysia* bag cell neurons and their axons (Blankenship and Nagle, unpub. data). This antibody is further characterized in this paper with regard to its binding to synthetic *Aplysia* ELH in dot immunoassays and its capability of binding to the caudodorsal cell (CDC) neurons and cerebral commissure of *L. stagnalis*. The CDC neurons and cerebral commissure contain the *L. stagnalis* peptide egg-laying neurohormone CDCH (Geraerts and Bohlken, 1976; Geraerts *et al.*, 1988). The N-terminal sequence of CDCH (LSITNDLRAIADSY-; Ebberink *et al.*, 1985) is

50% identical to the ELH sequence (Chiu *et al.*, 1979). Tests of positive staining of CDC neurons and cerebral commissure were used to determine whether the anti-ELH antibody had a sufficiently broad specificity that it would bind to CDCH, a homolog of *A. californica* ELH in a species from a different subclass of gastropods.

The primary anti-α-CDCP is a polyclonal rabbit serum obtained from J. Van Minnen (Vrije Universiteit, Amsterdam) and characterized in previous publications on *L. stagnalis* (Van Minnen *et al.*, 1992; Croll *et al.*, 1993). α-CDCP is biologically synthesized in *L. stagnalis* from the same precursor peptide as CDCH and is a member of a family of "alpha peptides" that are nine amino acids long and have an identical core sequence (amino acids 2 to 6, -PRLRF-) in *L. stagnalis* and *A. californica* (reviewed by Nagle *et al.*, 1989). Staining for α-CDCP-immunoreactivity was used to provide positive identification of CDC neurons and cerebral commissure in *L. stagnalis* and to determine whether immunoreactive alpha peptides were also present in ELH-immunoreactive cells in prosobranchs.

All of the immunohistological experiments reported here included, as positive controls, one or more *L. stagnalis* nervous systems carried through the same sectioning, staining, and micrographic procedures simultaneously with ganglia from other species. Negative control procedures included omitting the primary antibodies and preadsorbing the anti-ELH antibody with 5×10^{-6} mol/l synthetic *Aplysia* ELH (Peninsula Laboratories, Lot 012020).

Dot immunoassay

Synthetic *Aplysia* ELH was dissolved in deionized water at various concentrations, ranging from 0.01 μg to 2 μg/μl and applied in 1-μl dots on nitrocellulose membranes. After drying for at least 15 min, the nitrocellulose was immersed in PFAPB for 8–10 min, rinsed, and then incubated for 60 min at room temperature in nonspecific "blocker" solution (5% Carnation nonfat dry milk in TTBS [0.05% Tween 20, 500 m*M* NaCl, 20 m*M* Tris, pH 7.5]), with rocking. After the blocker solution was drained off, the membranes were incubated with rocking in anti-ELH (diluted 1:500 in blocker solution) at room temperature for 45–60 min. The membranes were then rinsed 2 times for 7 min in TTBS, incubated 45–60 min in secondary antibody (donkey anti-rabbit Ig horseradish peroxidase-linked $F(ab')_2$ fragment from Amersham, diluted 1:2000 in blocker solution), rinsed three times in TTBS, and then stored up to several hours in TBS (TTBS without Tween-20). The dot immunoassays were developed using a luminol-based enhanced chemiluminescence procedure (Pierce, SuperSignal Substrate). Immediately after completing the procedure, the location and intensity of the luminescing luminol produced by the reaction was determined by exposures to X-ray film for 5–10 min. Subsequent longer exposures (several hours) were used to confirm the presence of faint spots, while saturating the responses elsewhere.

Results

Anti-ELH immunoreactivity in Lymnaea stagnalis, *a positive control*

The antibody to *A. californica* ELH was tested first on nervous systems of *L. stagnalis*, a species from a different subclass of gastropods known to contain an ELH homolog, CDCH, in CDC neurons and the cerebral commissure. CDC neurons were identified by their characteristic clustering and positions, and were further positively identified using antibodies to α-CDCP (Fig. 2A). Few other cell bodies in the central nervous system bind to anti-α-CDCP, but some additional α-CDCP–immunoreactive

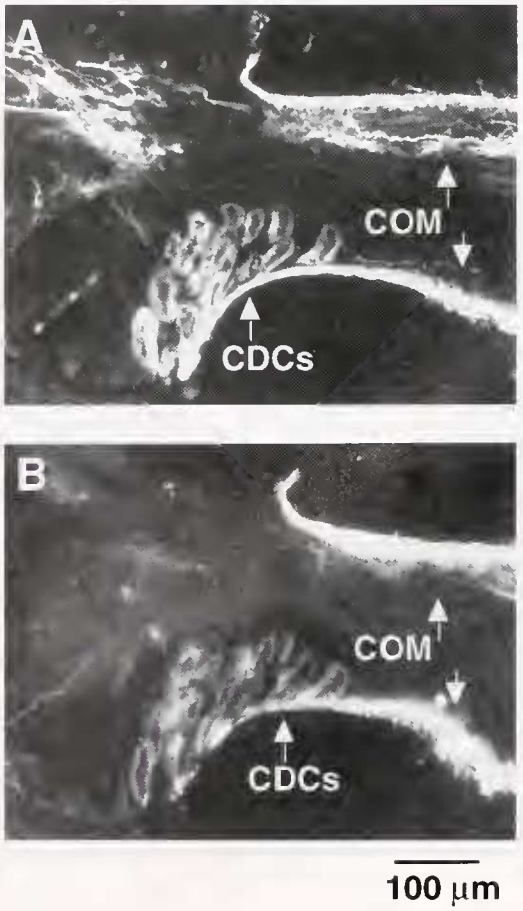

Figure 2. Immunoreactivity to anti-α-CDCP (A) and anti-ELH (B) in adjacent sections of a cerebral ganglion and cerebral commissure of *Lymnaea stagnalis*. Abbreviations: CDCs, caudodorsal cells; COM, cerebral commissure.

fibers were present as previously observed (Van Minnen *et al.*, 1992). Similarly, hormone-containing regions of the cerebral commissure were positively identified by their position and immunoreactivity to anti-α-CDCP. CDC neurons and cerebral commissure were then shown to be immunoreactive to anti-ELH. Figure 2B shows anti-ELH immunoreactivity in a section adjacent to the anti-α-CDCP–stained section in Figure 2A. Few other fibers or neurons were immunoreactive with the anti-ELH antibody, although a faint staining (much lighter than in the CDCs) of metacerebral giant cells was occasionally observed (not shown). This pattern of anti-ELH immunoreactivity was consistently obtained in *L. stagnalis* preparations that were processed in parallel with sections of ganglia from other species. Sections carried through the procedures in the absence of primary antibodies to ELH and α-CDCP, or with anti-ELH that had been preadsorbed with synthetic ELH, showed no staining with the secondary (FITC-labeled) antibody (not shown).

We also examined other ganglia of *L. stagnalis* for immunoreactive cells. As illustrated in Figure 3, the buccal ganglia each had a single neuron containing immunoreactive ELH and α-CDCP, consistent with a previous schematic representation of CDCH distribution (Geraerts *et al.*, 1988), but this is the first illustration of the location

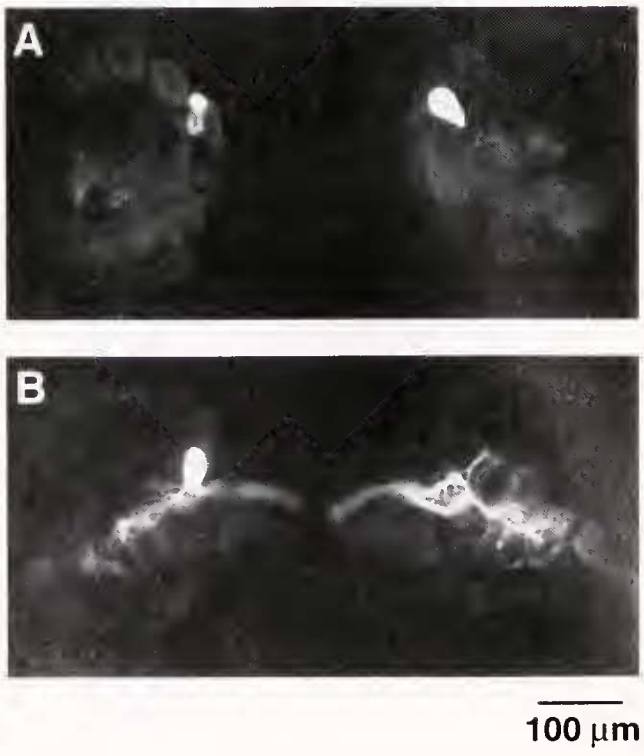

Figure 3. Pair of neurons and associated processes immunoreactive to anti-α-CDCP (A) and anti-ELH (B) in adjacent sections of buccal ganglia of *Lymnaea stagnalis*.

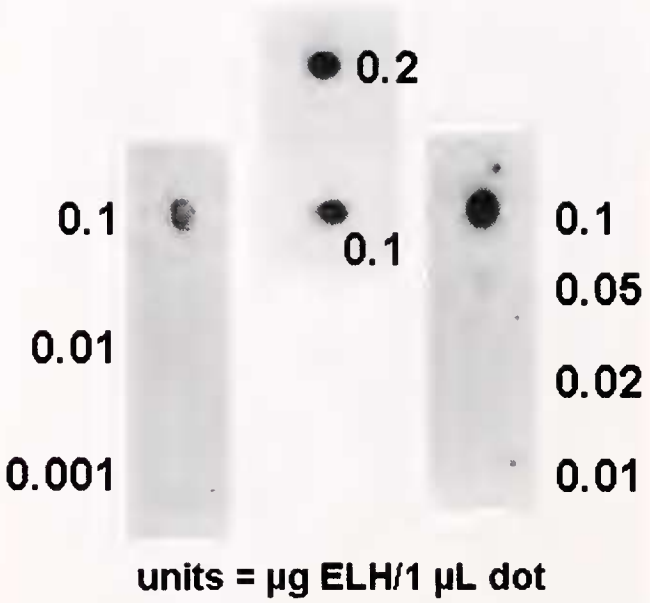

Figure 4. Dot immunoassays of synthetic *Aplysia* ELH, using anti-ELH. The number next to each dot indicates micrograms of ELH, applied to the paper in 1 µl.

and morphology of fibers of these buccal ganglion cells. Together, these findings demonstrate that the anti-ELH antibody employed here has a broad enough specificity to react with intracellular egg-laying neuropeptides in a species as distantly related as being in another gastropod subclass.

To determine whether the anti-ELH antibody could, in fact, bind to ELH and also to determine, to some degree, the sensitivity of its interaction, we conducted dot immunoassays with synthetic ELH. As illustrated in Figure 4, we could consistently ($n = 3$) detect as little as 0.1 µg ELH, with possible sensitivity down to 0.05 µg (slight response observed in one experiment). The antibody to α-CDCP did not bind to synthetic ELH.

Immunoreactive ELH and α-CDCP in Busycon canaliculatum

Cerebral ganglia of *B. canaliculatum* contain neurons immunoreactive to anti-ELH (Fig. 5A, C) and anti-α-CDCP (Fig. 5B, D, E). In serial sections through entire cerebral ganglia, both antibodies consistently labeled clusters of immunoreactive neurons that are located in bilaterally symmetric positions along the medial margins of the cerebral ganglia (Fig. 6). To ascertain that the antibodies were indeed staining the same cells, alternate sections from two preparations were stained with the two antibodies. In the example illustrated in Figure 5A, a large cluster of neurons stained well with the anti-ELH antibody. The same cluster also stained moderately,

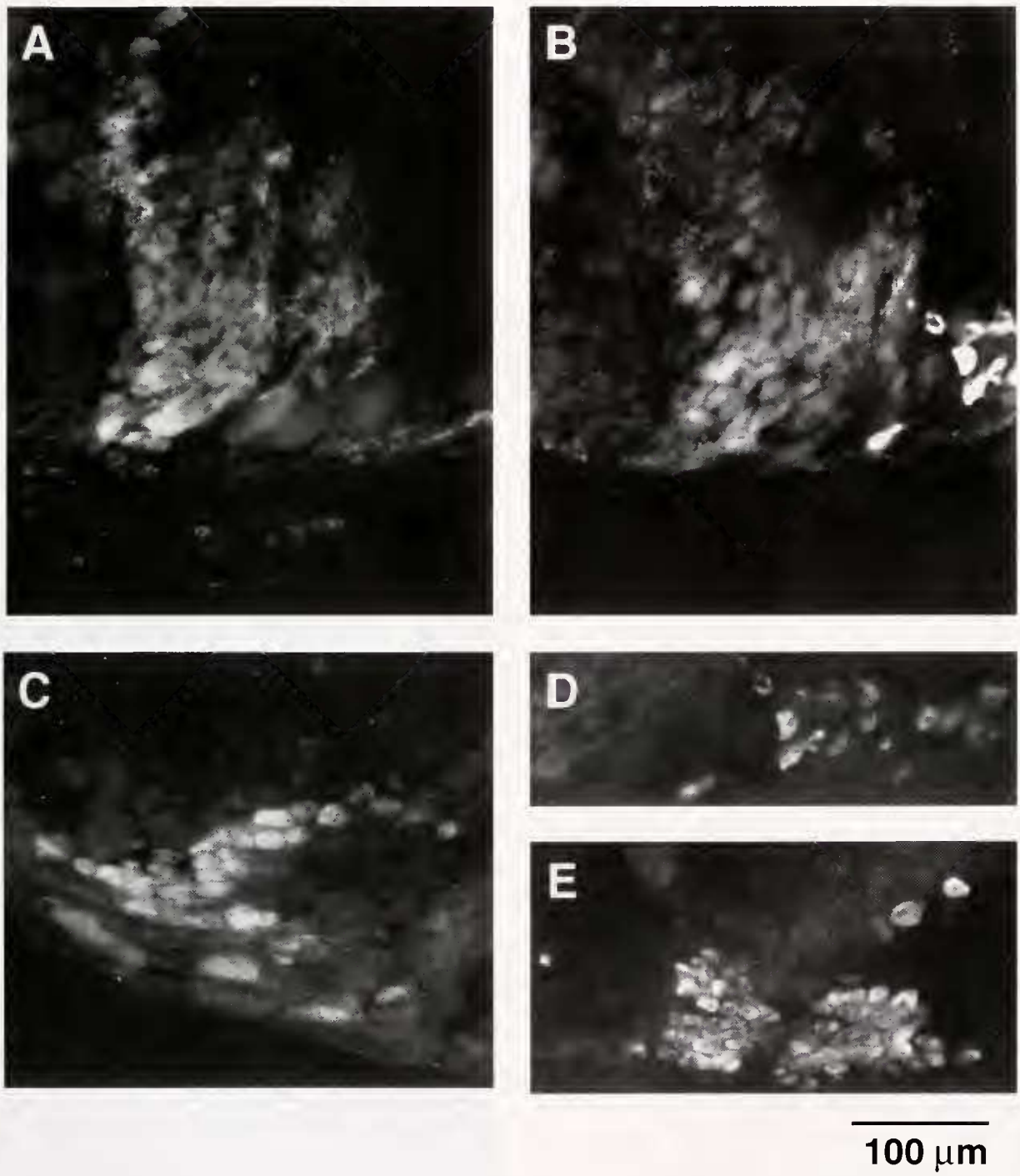

Figure 5. Immunoreactivity to anti-ELH (A, C) and anti-α-CDCP (B, D, E) in cerebral ganglia of *Busycon canaliculatum*. (A, B) Adjacent sections from one cerebral ganglion, showing staining of the same large cluster of cells with both (A) anti-ELH and (B) anti-α-CDCP antibodies. In addition, (B) shows part of a brighter cluster of anti-α-CDCP–immunoreactive cells at the lower right which has no counterpart in the anti-ELH micrograph. The entire cluster of these brighter cells from the same section as in (B) is shown in (D). (C) Anti-ELH–immunoreactive cell bodies from another cerebral ganglion. (E) Anti-α-CDCP–immunoreactive cell bodies from another cerebral ganglion.

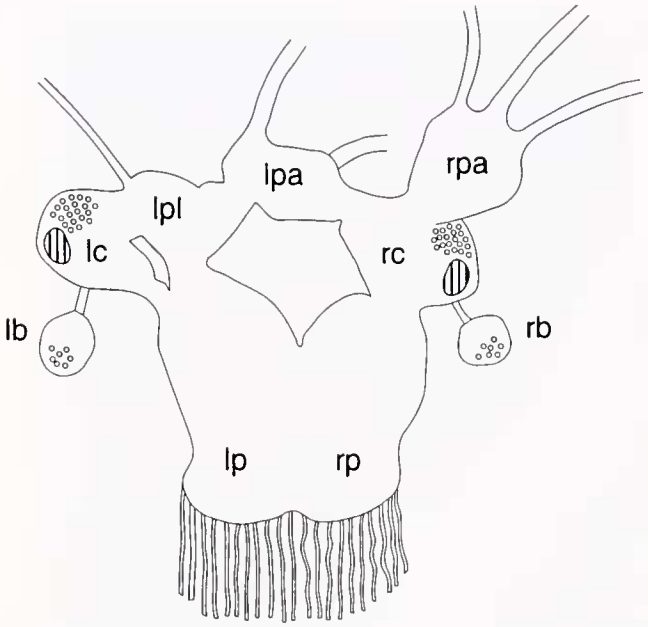

Figure 6. Schematic of locations of cells immunoreactive to anti-ELH and anti-α-CDCP in the nervous system of *Busycon canaliculatum*. The relative sizes and shapes of ganglia are shown for a preparation in which the circumesophageal ring of ganglia was opened by cutting through the cerebral commissure (cross-hatched areas of each cerebral ganglion). Small circles in the cerebral ganglia represent cells immunoreactive to both antibodies; circles in the buccal ganglia represent cell bodies of overlapping clusters of cells reactive to one or the other of the antibodies. Abbreviations: lb, left buccal; lc, left cerebral; lp, left pedal; lpa, left parietal; lpl, left pleural; rb, right buccal; rc, right cerebral; rp, right pedal; rpa, right parietal. Labeling of ganglia is according to Pierce (1950); however, the ganglia labeled as the left and right parietal ganglia probably correspond to ganglia usually described, respectively, as the subesophageal (or subintestinal) and supraesophageal (or supraintestinal) in most prosobranchs (Bullock, 1965).

though clearly above the background level of staining for many adjacent cells, with the anti-α-CDCP antibody (Fig. 5B). The anti-α-CDCP also stained more intensely a small, additional cell population in the region that did not bind the anti-ELH antibody (Fig. 5B, D). The other preparation stained with the antibodies on alternate sections showed a similar picture. In control experiments, alternate sections stained in the absence of primary antibody or with preadsorbed anti-ELH antibody showed no staining of cell bodies in cerebral ganglia.

Figures 5A and 5B were chosen to illustrate staining with anti-ELH and anti-α-CDCP of alternate sections of the same clusters, but they are not the most intense or clearly delineated examples we have of the appearance of the immunoreactive neurons. Accordingly, we illustrate clusters from a similar location in other cerebral ganglia in Figure 5C and Figure 5E. The diameters of the neuronal somata were in the range of 10–25 μm. Clusters of ELH-immunoreactive neurons comparable to those illustrated were generally found in 8 to 12 serial sections of each cerebral ganglion, with 30 to 60 stained cells per cluster on most of these sections. The average diameter of these cells is less than the thickness of each section (30 μm). Therefore, the total number of ELH-immunoreactive neurons per cerebral ganglion is estimated to be greater than 300.

Immunoreactive cell bodies were also seen in buccal ganglia of *B. canaliculatum* (Fig. 7). Immunoreactive ELH- and α-CDCP–containing neurons appeared in about the same location in these ganglia, but based on the number and sizes of the neurons, the two antibodies appear to be staining different subsets of neurons. In Figure 7, showing adjacent sections stained with the two antibodies, the α-CDCP–immunoreactive neurons appear to be smaller and more numerous than the anti-ELH–immunoreactive neurons. This pattern continued through several additional alternating sections (not shown).

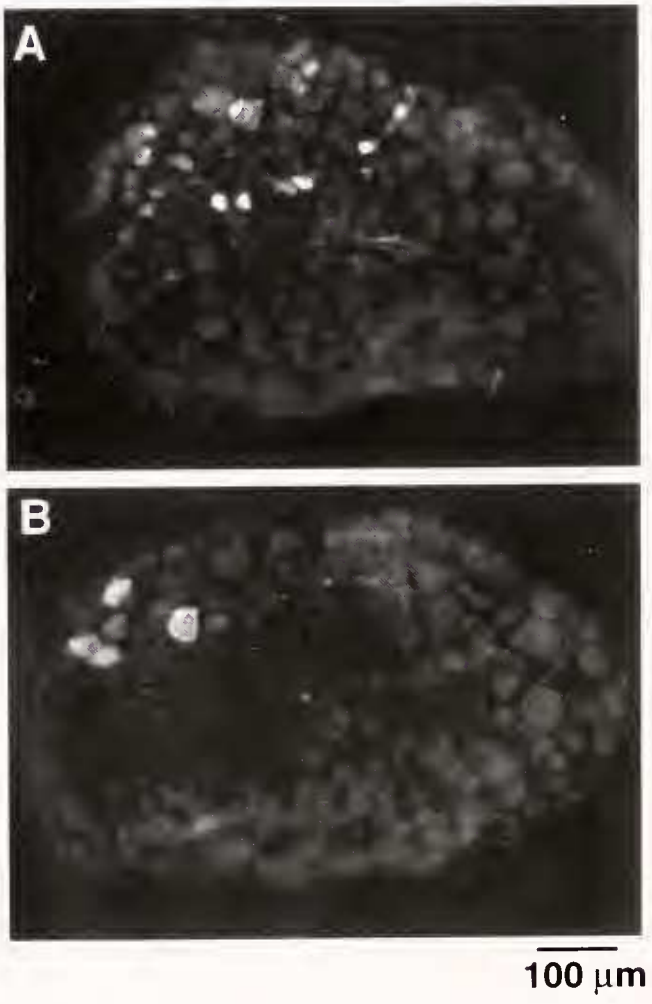

Figure 7. Immunoreactivity to anti-α-CDCP (A) and anti-ELH (B) in adjacent sections of a buccal ganglion of *Busycon canaliculatum*.

No immunoreactive cell bodies were seen in parietal and pleural ganglia. Although staining was observed in the surrounding connective tissue sheath (not shown), control sections stained without primary antibody also showed a significant amount of staining in the connective tissue sheath. Hence, the connective tissue staining in these ganglia might not reflect the presence of specific ELH immunoreactivity in this location. By comparison, there was no staining of cell bodies and relatively little staining of connective tissue in pedal ganglia.

Other prosobranch species

Anti-ELH immunoreactivity was also observed in *C. concholepas* (Fig. 8) and *T. atra* (Fig. 9). In both species, the cerebral ganglia were the only ganglia to show strongly immunoreactive cell bodies. In *C. concholepas*, compact clusters of more than 20 neurons each were present in the cerebral ganglia, whereas, in *T. atra*, numerous immunoreactive neurons appeared in several less compact clusters (*e.g.*, Fig. 9A shows neurons in two clusters). In *T. atra*, these clusters were on the surface of the ganglia and extended through a large number of sections.

T. atra is the only prosobranch in which we have seen unambiguous anti-ELH immunoreactivity in fibers. Figure 9A clearly illustrates several neuronal cell bodies from which well-stained processes originate. In addition, staining of single fibers (Fig. 9B) and tracts of fibers (Fig. 9C) were consistently (three different preparations) seen in the cerebral-pedal connectives of *T. atra*. These fibers could not be traced back to their cell bodies of origin. They were present almost all of the way from the cerebral ganglia to the pedal ganglia, but no positively stained cell bodies or fibers were seen in the pedal ganglia. When single fibers could be seen in the nerves, the fibers always had a varicose appearance with a diameter less than 0.5 μm.

Staining of sections of the above preparations from *C. concholepas* and *T. atra* for anti-α-CDCP immunoreactivity gave negative results.

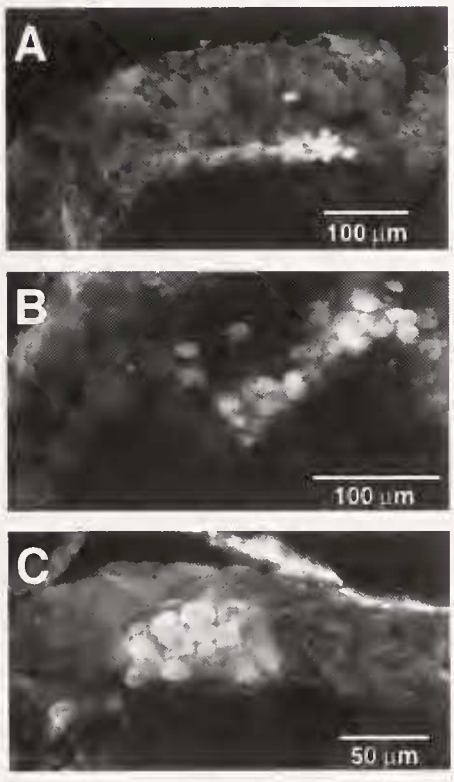

Figure 8. Anti-ELH–immunoreactive cell bodies in cerebral ganglia from three specimens of *Concholepas concholepas*.

Discussion

We report here the first use of immunocytochemistry to localize ELH- and α-CDCP-like peptides in prosobranchs. The anti-ELH antibody used in this study is shown to bind to low concentrations of synthetic ELH and to have a broad species range. The localization of anti-ELH immunoreactivity in cerebral ganglia of three different prosobranch species supports these ganglia as a major locus of peptides that regulate reproduction in this subclass of gastropods. The antibodies used in this study may therefore be useful in future studies to purify prosobranch ELH-like peptides, to study the physiological properties of their secretory neurons, and to identify the location and function of these neurons in other molluscan classes and species.

Observations of immunoreactivity to anti-ELH and anti-α-CDCP support the cerebral ganglia as candidates for the location of secretory neurons regulating egg laying in prosobranchs. The positive control data obtained here in *L. stagnalis* (Figs. 2 and 3) clearly confirm that both antibodies stain the CDC neurons, the cerebral commissure, and a pair of neurons in the buccal ganglia, previously reported to contain CDCH, the egg-laying hormone of *L. stagnalis* (Geraerts *et al.*, 1988). In *B. canaliculatum*, a population of neurons immunoreactive to both anti-ELH and anti-α-CDCP antibodies was found in the cerebral ganglia (Figs. 5A, B and 6). Since cerebral ganglia also produced positive laying bioassays, these neurons are the most likely candidates for the source of the peptide causing this response. A cerebral ganglion localization of ELH-like immunoreactivity was also observed in the top snail *T. atra*. Although no bioassay experiments with *T. atra* have been reported, experiments in the related top snail species *Gibbula* spp. and *M. lineata* demonstrated that spawning could be elicited by cerebral ganglion extracts (Clare, 1986, 1987). ELH-like immunoreactive neurons found in *T. atra* are therefore good candidates for the source of this peptide as well. Likewise,

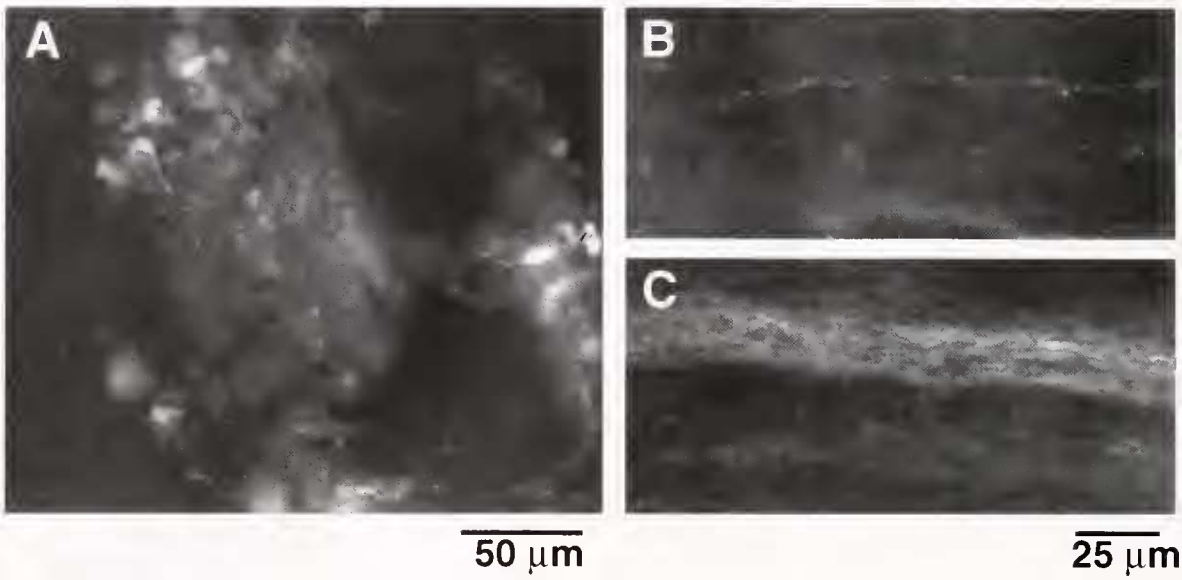

Figure 9. Anti-ELH–immunoreactive cell bodies and fibers in the nervous system of *Tegula atra*. (A) Section of a cerebral ganglion showing immunoreactive cell bodies from two clusters. Several of the neurons also have clearly evident immunoreactive axons. (B, C) Axons in the cerebral-pedal connective: (B) a single fiber; (C) a tract of immunoreactive fibers. The calibration mark at the right is for both (B) and (C).

C. concholepas ELH-like immunoreactivity was found in cerebral ganglia, which are therefore good candidates for secretory neurons controlling egg laying in that species. Among opisthobranchs, extracts of cerebral ganglia of *Archidoris montereyensis* can produce egg laying (Wiens and Brownell, 1994), and small clusters of ELH-immunoreactive neurons are present in cerebral ganglia of *A. californica* (Chiu and Strumwasser, 1984). Thus, according to the present data, a major locus of the secretory neurons regulating egg laying in prosobranchs, as in pulmonates, appears to be cerebral ganglia, and the present data provide a more precise anatomical localization for the cell bodies of these neurons than previous bioassay experiments.

In addition to cerebral ganglia, ELH-like immunoreactivity was also found elsewhere. The buccal ganglia of both *B. canaliculatum* and *L. stagnalis* contained immunoreactive neurons. ELH expression has been reported in buccal ganglia of *A. californica* in immature animals (McAllister *et al.*, 1983; Pulst *et al.*, 1988) but is apparently absent from buccal ganglia of mature *A. californica* (Chiu and Strumwasser, 1984; Pulst *et al.*, 1988). In *B. canaliculatum*, buccal ganglia do not elicit spawning upon bioassay with the amounts of extracts usually tested (*e.g.*, Ram, 1977), possibly indicating that the small number of putative ELH-containing neurons do not contain a large enough amount of the peptide to elicit egg laying. In *A. californica*, ELH is known to have direct effects on neurons, including neurons in buccal ganglia (Ram and Ram, 1989). By analogy, ELH in buccal ganglia of *B. canaliculatum* and *L. stagnalis* could be acting on local circuits, rather than being released for hormonal activation of egg laying.

This study did not clearly localize ELH-like immunoreactivity in parietal and pleural ganglia of *B. canaliculatum*. Bioassays of extracts of individual parietal and pleural ganglia elicited laying as reliably as did cerebral ganglia (Ram, 1977). Yet, no immunoreactive cell bodies were found in parietal and pleural ganglia. Only the connective tissue sheath was stained, and it could not be distinguished from nonspecific staining of the same tissue in control sections. Possibly the connective tissue sheath of the these ganglia is the site of neurohaemal secretion of ELH. Further studies to reduce the nonspecific staining in this region and to identify immunoreactive structures, if any, may help resolve this problem.

With regard to the targets of the anti-ELH antibody, dot immunoassays show, as expected, that the anti-ELH antibody binds to low concentrations of synthetic ELH. It has been estimated that the bag cells of a mature *A. californica* contain approximately 20 μg ELH (Chiu *et al.*, 1979). The dot immunoassays reliably detected 0.1 μg ELH, approximately 1/200th the amount in a mature animal and much less than the amount detectable by bioassay (approximately ½ of the hormone in a bag cell cluster; Toevs, 1970). In other species, where the antigenic peptides may not provide a perfect fit for the antibody, the sensitivity may be much less, but even a 10-fold decrease in the detectable concentration would make it a much more sensitive technique for detecting ELH than bioassays in congeneric species.

Although ELH-like immunoreactivity was observed in all three species of prosobranchs that were examined, anti-α-CDCP staining was obtained only in *B. canaliculatum* and in the positive control pulmonate, *L. stagnalis*. The negative results for α-CDCP-like immunoreactivity in *C. concholepas* and *T. atra* could have a number of explanations. These include lack of these peptides in these species, presence of α-CDCP homologs that are sufficiently dissimilar to α-CDCP to lack immunoreactivity to the serum used, and loss of immunoreactivity due to the differences in fixation of these Chilean specimens. Physiological experiments have indicated that alpha peptides may have an autoregulatory function in neurons that secrete ELH or CDCH (Rothman *et al.*, 1983; Brussaard *et al.*, 1990; Redman and Berry, 1991). Therefore, determination of the presence, location, and structural or functional differences of alpha peptides in prosobranchs may be important in understanding adaptations for regulating secretion of the ELH-like peptides in prosobranchs.

Further studies identifying the peptides to which these antibodies bind in the prosobranchs are needed, to determine whether they are indeed related structurally to ELH/CDCH and the alpha peptides, to measure the sensitivity with which they can be detected with these techniques, and to ascertain their relationship to the substances in nervous system extracts previously shown to cause egg-laying in prosobranch species. Nevertheless, the results shown in this paper demonstrate that antibodies to pulmonate and opisthobranch reproductive peptides bind to specific groups of neurons in cerebral and other ganglia of prosobranchs, identifying these neurons for future functional studies.

Acknowledgments

This research was supported, in part, by a Dalhousie Medical Research Foundation Picchione Visiting Scholarship and NSF grant INT-9724918 to J. L. Ram, by grants from Fondecyt (1960488) and the Volkswagen Foundation (I 96-01) to C. S. Gallardo, and an NSERC (Canada) Strategic Grant to R. P. Croll. The authors gratefully acknowledge Dr. Ariel Pinto, of the Instituto de Fomento Pesquero, Castro, Chile, for specimens and information regarding egg-laying behavior of *Concholepas concholepas*, and Janette Nason for help with histology. Antibodies were obtained from J. E. Blankenship and G. T. Nagle (anti-ELH) and J. Van Minnen (anti-α-CDCP). Dedicated to the memory of Sharon L. Ram.

Literature Cited

Brussaard, A. B., N. C. M. Schluter, R. H. M. Ebberink, K. S. Kits, and A. ter Maat. 1990. Discharge induction in molluscan peptidergic cells requires a specific set of autoexcitatory neuropeptides. *Neuroscience* **39:** 479–491.

Bullock, T. H. 1965. Mollusca: Gastropoda. Pp. 1283–1386 in *Structure and Function in the Nervous Systems of Invertebrates, Volume II*, T. H. Bullock and G. A. Horridge, eds. W. H. Freeman, San Francisco.

Castagna, M., and J. N. Kraeuter. 1994. Age, growth-rate, sexual dimorphism and fecundity of knobbed whelk *Busycon carica* (Gmelin, 1791) in a western mid-Atlantic lagoon system, Virginia. *J. Shellfish Res.* **13:** 581–585.

Castilla, J. C. 1988. Una revisión bibliográfica (1980–1988) sobre *Concholepas concholepas* (Gastropoda, Muricidae): Problemas pesqueros y experiencia de repoblación. *Biol. Pesq. (Chile)* **17:** 9–19.

Castilla, J. C., and J. Cancino. 1976. Spawning behavior and egg capsules of *Concholepas concholepas* (Mollusca: Gastropoda: Muricidae). *Mar. Biol.* **37:** 255–263.

Chiu, A. Y., and F. Strumwasser. 1984. Two neuronal populations in the head ganglia of *Aplysia californica* with egg-laying hormone-like immunoreactivity. *Brain Res.* **294:** 83–93.

Chiu, A. Y., M. W. Hunkapiller, E. Heller, D. K. Stuart, L. E. Hood, and F. Strumwasser. 1979. Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **76:** 6656–6660.

Clare, A. S. 1986. Induction of egg spawning in *Gibbula umbilicalis* (da Costa) by an homogenate of the cerebral ganglia. *Gen. Comp. Endocrinol.* **64:** 85–90.

Clare, A. S. 1987. Studies on the putative egg-spawning neurohormones of *Gibbula umbilicalis* (da Costa) and *G. cineraria* (L.). *Gen. Comp. Endocrinol.* **66:** 48–49.

Collin, R. 1995. Sex, size, and position: a test of models predicting size at sex change in the protandous gastropod *Crepidula fornicata*. *Am. Nat.* **146:** 815–831.

Coloma, S. L. 1974. Estudio histológico de la gónada de *Tegula atra*. *Bol. Soc. Biol. Concepción* **48:** 359–363.

Croll, R. P., and B. J. Chiasson. 1989. Post-embryonic development of serotonin-like immunoreactivity in the central nervous system of the snail, *Lymnaea stagnalis*. *J. Comp. Neurol.* **280:** 122–142.

Croll, R. P., J. Nason, and J. van Minnen. 1993. Characterization of neurons in bivalves using antibodies raised against neuropeptides involved in the control of egg-laying in gastropods. *Invertebr. Reprod. Dev.* **24:** 161–168.

De Vlieger, T. A., K. S. Kits, A. ter Maat, and J. C. Lodder. 1980. Morphology and electrophysiology of the ovulation hormone producing neuro-endocrine cells of the freshwater snail *Lymnaea stagnalis* (L.). *J. Exp. Biol.* **84:** 259–271.

DiSalvo, L., and M. R. Carriker. 1994. Planktonic metamorphic and early benthic behavior of the Chilean loco *Concholepas concholepas* (Muricidae, Gastropoda, Mollusca). *J. Shellfish Res.* **13:** 57–66.

Ebberink, R. H. M., H. van Loenhout, W. P. M. Geraerts, and J. Joosse. 1985. Purification and amino acid sequence of the ovulation neurohormone of *Lymnaea stagnalis*. *Proc. Natl. Acad. Sci. USA* **82:** 7767–7771.

Gallardo, M. H., and J. I. Carrasco. 1996. Genetic cohesiveness among populations of *Concholepas concholepas* (Gastropoda, Muricidae) in southern Chile. *J. Exp. Mar. Biol. Ecol.* **197:** 237–249.

Geraerts, W. P. M., and S. Bohlken. 1976. The control of ovulation in the hermaphrodite freshwater snail *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.* **28:** 350–357.

Geraerts, W. P. M., A. ter Maat, and E. Vreugdenhil. 1988. The peptidergic neuroendocrine control of egg-laying behavior in *Aplysia* and *Lymnaea*. Pp. 141–231 in *Invertebrate Endocrinology, Endocrinology of Selected Invertebrate Types*, H. Laufer and G. H. Downer, eds. Alan R Liss, New York.

Haszprunar, G. 1988. On the origin of major gastropod groups, with special reference to the Streptoneura (Mollusca). *J. Molluscan Stud.* **54:** 367–441.

Huaquin, L. 1966. Anatomia de *Concholepas concholepas* (Bruguière, 1789). Thesis. Facultad de Medicina, Universidad Catolica de Chile, Santiago, Chile. 53 pp.

Hyman, L. H. 1967. Pp. 247–262 in *The Invertebrates. Vol. VI, Mollusca.* McGraw-Hill, New York.

Jansen, R. F., and A. ter Maat. 1985. Ring neuron control of columellar motor neurons during egg-laying behavior in the pond snail. *J. Neurobiol.* **16:** 1–14.

Kaplan, J. M., and B. C. Boyer. 1992. Monitoring marine resources: ecological and policy implications affecting the scientific collecting and commercial value of New England conch (*Busycon*). *Biol. Bull.* **183:** 379–380.

Kupfermann, I. 1967. Stimulation of egg-laying: possible neuroendocrine functions of bag cells of abdominal ganglion of *Aplysia californica. Nature* **216:** 814–815.

McAllister, L. B., R. H. Scheller, E. R. Kandel, and R. Axel. 1983. *In situ* hybridization to study the origin and fate of identified neurons. *Science* **222:** 800–808.

Nagle, G. T., S. D. Painter, and J. E. Blankenship. 1989. Post-translational processing in model neuroendocrine systems: precursors and products that coordinate reproductive activity in *Aplysia* and *Lymnaea. J. Neurosci. Res.* **23:** 359–370.

Osorio, C. 1979. Moluscos marinos de importancia económica en Chile. *Biol. Pesq. (Chile)* **11:** 3–47.

Pierce, M. E. 1950. *Busycon canaliculatum.* Pp. 336–344 in *Selected Invertebrate Types,* F. A. J. Brown, ed. John Wiley, New York.

Pulst, S.-M., D. Gusman, and E. Mayeri. 1988. Immunostaining for peptides of the egg-laying hormone/bag cell peptide precursor protein in the head ganglia of *Aplysia. Neuroscience* **27:** 363–371.

Ram, J. L. 1977. Hormonal control of reproduction in *Busycon:* laying of egg capsules caused by nervous system extracts. *Biol. Bull.* **152:** 221–232.

Ram, J. L., and M. L. Ram. 1989. Synthetic egg-laying hormone of *Aplysia:* quantitative studies of induction of egg laying in *Stylocheilus* and of the activation of *Aplysia* buccal neuron B16. *Comp. Biochem. Physiol.* **92C:** 131–134.

Ram, J. L., and M. L. Ram. 1990. Gastropod egg-laying hormones. Pp. 257–264 in *Advances in Invertebrate Reproduction,* M. Hoshi and O. Yamashita, eds. Elsevier, Amsterdam.

Ram, J. L., M. L. Ram, and J. P. Davis. 1982. Hormonal control of reproduction in *Busycon:* II. Laying of egg-containing capsules caused by nervous system extracts and further characterization of the substance causing capsule-laying. *Biol. Bull.* **162:** 360–370.

Redman, R. S., and R. W. Berry. 1991. Temperature-dependent peptidergic feedback: potential role in seasonal egg laying in *Aplysia. J. Neurosci.* **11:** 1780–1785.

Rodriguez, S. R., C. Riquelme, E. O. Campos, P. Chavez, E. Brandan, and N. C. Inestrosa. 1995. Behavioral responses of *Concholepas concholepas* (Bruguiére, 1789) larvae to natural and artificial settlement cues and microbial films. *Biol. Bull.* **189:** 272–279.

Rothman, B. S., E. Mayeri, R. O. Brown, P.-M. Yuan, and J. E. Shively. 1983. Primary structure and neuronal effects of alpha-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia. Proc. Natl. Acad. Sci. USA* **80:** 5753–5757.

Serra, G., G. Chelazzi, and J. C. Castilla. 1997. Effects of experience and risk of predation on the foraging behavior of the Southeastern Pacific muricid *Concholepas concholepas* (Mollusca: Gastropoda). *J. Anim. Ecol.* **66:** 876–883.

Solem, G. A. 1991. Gastropods (snails and slugs). Pp. 311–321 in *The New Encyclopedia Britanica, 15th ed.* Encyclopedia Britannica, Chicago.

Toevs, L. 1970. Identification and characterization of the egg-laying hormone from the neurosecretory bag cells of *Aplysia.* Ph.D. Dissertation. Division of Biology, California Institute of Technology, Pasadena, California.

Toevs, L., and R. W. Brackenbury. 1969. Bag cell specific proteins and the humoral control of egg laying in *Aplysia californica. Comp. Biochem. Physiol.* **29:** 207–216.

Van Minnen, J., H. D. F. H. Schallig, and M. D. Ramkema. 1992. Identification of putative egg-laying hormone containing neuronal systems in gastropod molluscs. *Gen. Comp. Endocrinol.* **86:** 96–102.

Wiens, B. L., and P. H. Brownell. 1994. Neuroendocrine control of egg-laying behavior in the nudibranch, *Archidoris montereyensis. J. Comp. Neurol.* **344:** 619–625.